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# AMPA Receptor Subunit-Specific Regulation by a Distinct Family of Type II TARPs

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DOI 10.1016/j.neuron.2008.07.034

## SUMMARY

**AMPA-type glutamate receptors (GluRs) play major roles in excitatory synaptic transmission. Neuronal AMPA receptors comprise GluR subunits and transmembrane AMPA receptor regulatory proteins (TARPs). Previous studies identified five mammalian TARPs, γ-2 (or stargazin), γ-3, γ-4, γ-7, and γ-8, that enhance AMPA receptor function. Here, we classify γ-5 as a distinct class of TARP that modulates specific GluR2-containing AMPA receptors and displays properties entirely dissimilar from canonical TARPs. γ-5 increases peak currents and decreases the steady-state currents selectively from GluR2-containing AMPA receptors. Furthermore, γ-5 increases rates of GluR2 deactivation and desensitization and decreases glutamate potency. Remarkably, all effects of γ-5 require editing of GluR2 mRNA. Unlike other TARPs, γ-5 modulates GluR2 without promoting receptor trafficking. We also find that γ-7 regulation of GluR2 is dictated by mRNA editing. These data establish γ-5 and γ-7 as a separate family of "type II TARPs" that impart distinct physiological features to specific AMPA receptors.**

## INTRODUCTION

Glutamate serves as the major excitatory neurotransmitter in brain. Fast transmission at glutamate synapses is mediated by AMPA-, NMDA-, and kainate-type ion channels. The AMPA subtype induces most of the postsynaptic depolarization that leads to neuronal firing. This moment-to-moment signaling by AMPA receptors contributes fundamentally to sensory, motor, and integrative brain functions. Furthermore, activity-dependent changes in synaptic AMPA receptor levels contribute to the plasticity that underlies aspects of learning and memory (Bredt and Nicoll, 2003; Malinow and Malenka, 2002; Sheng and Kim, 2002; Song and Huganir, 2002).

AMPA receptors comprise homo- and heterotetramers of GluR1-4 subunits, which contain both the glutamate-binding and ion channel structures (Hollmann and Heinemann, 1994; Seeburg, 1993). These subunits show distinct ion channel conductances and kinetic properties. Alternative splicing, which yields flip and flop forms for each GluR subunit, further amplifies this diversity (Sommer et al., 1990). Importantly, editing of the GluR2 mRNA changes a crucial Gln (Q) to Arg (R) in the ion channel pore region such that GluR2-containing AMPA receptors permeate only monovalent cations, whereas GluR2-lacking receptors also conduct $Ca^{2+}$ (Hollmann et al., 1991; Sommer et al., 1991). Some studies suggest that "subunit-specific rules" differentially control AMPA receptor trafficking and synaptic plasticity (Lee et al., 2004; Malinow and Malenka, 2002; Song and Huganir, 2002). Supporting these models for subunit-specific regulation of trafficking, certain binding partners for AMPA receptors associate specifically with GluR1 or GluR4 subunits, which have long cytoplasmic tails, whereas other interacting proteins bind to GluR2 or GluR3 subunits, which have short cytoplasmic tails.

In addition to the GluR principal subunits, neuronal AMPA receptors also contain TARP auxiliary subunits (Nicoll et al., 2006; Vandenberghe et al., 2005b). Mutation of stargazin (or γ-2), the prototypical TARP, yields stargazer mice, which suffer absence epilepsy and cerebellar ataxia (Letts et al., 1998). This ataxia is associated with selective loss of AMPA receptor function in cerebellar granule cells (Chen et al., 2000; Hashimoto et al., 1999). Cellular studies of stargazer granule cells showed that stargazin escorts AMPA receptors through the secretory pathway to the synapse (Chen et al., 2000; Vandenberghe et al., 2005a). In addition to trafficking AMPA receptors, stargazin also modulates receptor pharmacology and controls channel gating. Specifically, stargazin increases AMPA receptor glutamate affinity, enhances single-channel conductance, slows deactivation and desensitization, and reduces the extent of desensitization (Bedoukian et al., 2006; Priel et al., 2005; Tomita et al., 2005; Turetsky et al., 2005). Furthermore, a family of three other highly homologous TARPs, γ-3, γ-4, and γ-8, regulate AMPA receptors in distinct cell types throughout the brain (Tomita et al., 2003). These four TARPs all show qualitatively similar regulation of all four GluR isoforms; however, distinct TARP subunits show differences in their modulation of channel gating. Specifically, γ-4 shows the quantitatively greatest effect to slow AMPA receptor gating (Cho et al., 2007; Korber et al., 2007b; Kott et al., 2007; Milstein et al., 2007).

TARPs were originally named "γ-subunits" based on their sequence homology with the γ-1 tetraspanning calcium channel subunit (Jay et al., 1990), which occurs only in skeletal and cardiac muscles and does not regulate AMPA receptors (Tomita et al., 2003). The extended γ-subunit family contains the four canonical TARPs, as well as γ-1, -5, -6, and -7. Our original studies showed that γ-1 and γ-5 do not augment trafficking or gating of GluR1 (Tomita et al., 2003). Whereas γ-1 and γ-6 occur largely in striated skeletal muscle, we recently reported that the highly

homologous γ-7 and γ-5 proteins are enriched in brain and that γ-7—but not γ-5—greatly enhances glutamate-evoked currents from GluR1 (Kato et al., 2007). Further, we found that γ-7—but not γ-5—partially restores functional AMPA receptors on stargazer cerebellar granule cells.

Here, we examine possible roles for γ-5 in regulating AMPA receptors. Using an optimized immunoprecipitation procedure, we demonstrate that γ-5 associates with several GluR subunits in solubilized brain extracts. In heterologous cells, we find a unique subunit specificity for γ-5 in regulating GluR channel activity. That is, γ-5 enhances glutamate-evoked currents only from certain GluR2-containing receptors. The critical mRNA-edited Q/R site in the pore of GluR2 mediates this remarkable specificity. γ-5 also uniquely and greatly *lowers* glutamate affinity and markedly *suppresses* steady-state currents, especially from GluR2-containing receptors. Interestingly, γ-5 mediates all these regulatory effects without enhancing cellular trafficking of GluR subunits. The highly homologous γ-7 subunit shares some of these properties with γ-5. These studies define γ-5 and γ-7 as a subfamily of type II TARPs as compared to the type I TARPs, γ-2, γ-3, γ-4, and γ-8. This dichotomy of TARP function adds to the diversity of AMPA receptor physiology and plasticity.

## RESULTS

Previous studies showed that γ-5 is weakly and specifically bound to GluR1 in cerebellar extracts (Kato et al., 2007). Here, we optimized the immunoprecipitation procedure by increasing the amounts and concentrations of solubilized membrane proteins and antibodies. This improved procedure showed specific binding of γ-5 with GluR1, GluR2/3, and GluR4 in cerebellar extracts (Figure 1A). We previously showed that γ-5 in cerebellum does not occur in the PSD (Kato et al., 2007), which could reflect the enrichment of γ-5 in cerebellar Bergman glia that lack PSD domains. To determine whether γ-5 in forebrain neurons might be enriched in the PSD, we fractionated membranes from olfactory bulb. We find that γ-5 in these preparations did partially enrich in the PSD and showed a similar fractionation as γ-2, -3, -4, and -8 (Figure 1B). As our anti-γ-5 antibody is not suitable for immunostaining of endogenous γ-5, we overexpressed γ-5 in cultured cortical neurons and coimmunostained with anti-PSD-95. Strong γ-5 signals were observed in somatic regions, and γ-5 was also concentrated in puncta along the neurites. Many of these γ-5 puncta colocalized with PSD-95 (see Figure S1 available online), implying a synaptic distribution.

### Subunit-Specific Regulation of GluR by Type II TARPs

We next used whole-cell recordings from transfected HEK293 cells to evaluate regulation of AMPA receptors by γ-5. Individual cells were captured and perfused with a 16-barrel pipette array, which allowed for rapid sequential titration of pharmacological reagents. In HEK293 cells transfected with the flip form of GluR1 alone, glutamate application elicited a peak current of ~2000 pA, which rapidly decayed to a steady-state current of ~20 pA (Figure 2A). As previously reported (Tomita et al., 2003), cotransfection with γ-5 did not significantly alter either the peak or steady-state currents evoked from GluR1. By contrast, cotransfection of GluR1 with γ-2 or γ-7 augmented both

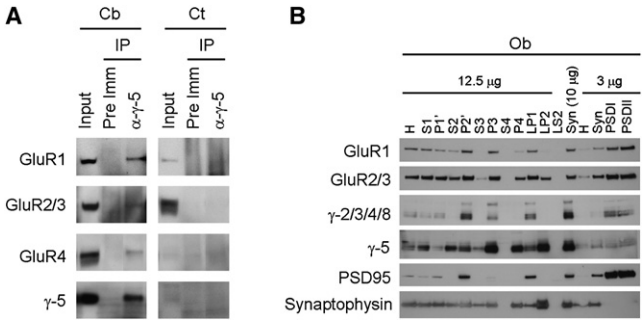


**Figure 1. γ-5 Associates with Neuronal AMPA Receptors**

(A) Cerebellar (Cb) or cerebrocortical (Ct) membranes were solubilized with Triton X-100 (0.1%) and immunoprecipitated (IP) with preimmune IgG or anti-γ-5 antibody, and bound proteins were immunoblotted with anti-GluRs or anti-γ-5 antibodies.

(B) Subcellular fractionation of rat olfactory bulb. γ-5 is enriched in P3, P4, LP2, and synaptosomal fractions and also occurs in the PSD. The distribution resembles that of γ-2/3/4/8 and GluR1/2/3, whereas PSD-95 is enriched in and synaptophysin is absent from PSD.

the peak and steady-state currents (Figure 2A). As previously published (Kato et al., 2007), the glutamate-evoked currents from cells expressing GluR1 and γ-7 showed an unusual kinetic course comprising an initial peak and desensitization followed by a slower "run-up" in conductance to reach steady-state (Figure 2B). For these and all studies, we found that γ-6, a striated muscle protein that shares highest homology with γ-1, was without effect on glutamate-evoked currents.

In contrast to the lack of effect of γ-5 on GluR1 responses, coexpression of γ-5 with GluR2 markedly enhanced the peak current from these receptors (Figures 2A and 3). This enhancement of peak current by γ-5 was coupled with a nearly complete elimination of steady-state current from GluR2 (Figures 2A, 2B, and 3). The augmentation of GluR2 glutamate-evoked peak current and reduction of steady-state current reveals an unappreciated role for γ-5 as a TARP isoform that *enhances* the extent of AMPA receptor desensitization. In comparison, γ-2 and γ-7 exhibited classical TARP activity by augmenting both the peak and the steady-state glutamate-evoked currents from GluR2 (Figures 2A, 2B, and 3). Of note, the effects of γ-7 on GluR2 were similar to its effects on GluR1 such that there was an enhancement of peak current followed by rapid desensitization and a subsequent gradual increase to steady-state current (Figure 2B).

We further evaluated the effects of γ-2, γ-5, and γ-7 on GluR3 and GluR4. As previously published, γ-2 enhanced both the peak and steady-state currents from cells transfected with GluR3 or GluR4 (Figure 3). In contrast, γ-5 and γ-7 had no effects on the peak current of GluR4 and even reduced that of GluR3. For steady-state currents, γ-5 had minimal effects whereas γ-7 increased steady-state currents from GluR3, but not GluR4.

Because of the extremely rapid desensitization of AMPA receptors, the maximal currents measured in our experiments underestimate the true peak current. To better assess the effects of TARPs on the maximal peak glutamate-evoked currents, we added cyclothiazide (CTZ), which blocks desensitization of the

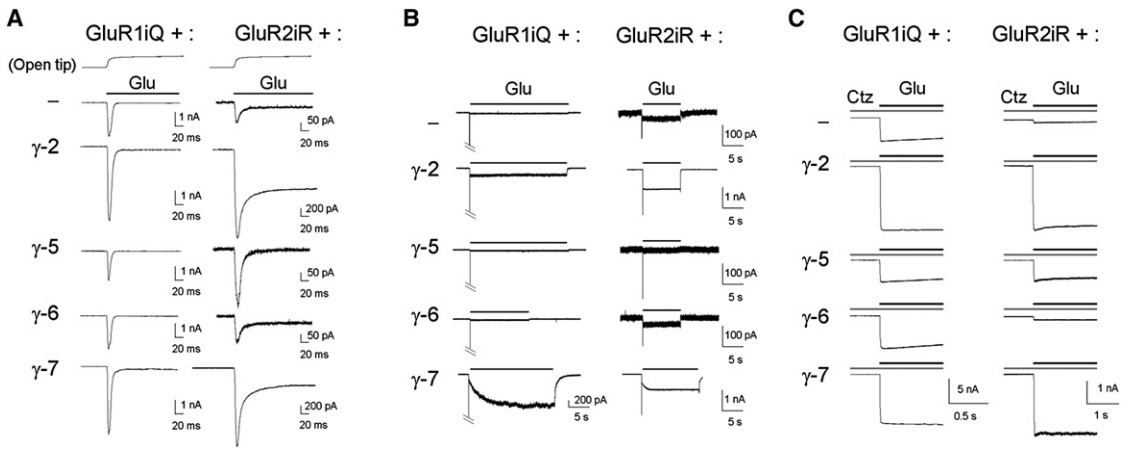


**Figure 2. γ-5 Modulates GluR2 but Not GluR1 Homomeric AMPA Receptors**

(A and B) Glutamate-evoked whole-cell currents from HEK293T cells transfected with GluR1 or GluR2 alone or with γ-2, γ-5, γ-6, or γ-7. (A) γ-5 increased glutamate-evoked peak currents from GluR2iR, but not those from GluR1iQ. γ-2 and γ-7 increased the currents of both GluR2iR and GluR1iQ. γ-6 had no effects on either GluR2iR or GluR1iQ. Open-tip recording determined the speed of perfusate switching. Extended time scale (B) shows that γ-5 reduced the steady-state currents from GluR2iR evoked by glutamate but had no effect on steady-state currents from GluR1iQ. γ-2 and γ-7 increased currents from both GluR2iR and GluR1iQ.

(C) Currents evoked by glutamate (1 mM) in the presence of CTZ (40 μM). γ-5 increased the currents from GluR2iR but not GluR1iQ.

flip isoform of GluR subunits. Consistent with previous findings (Kott et al., 2007), γ-2 enhanced the responses of GluR1-4 to glutamate in the presence of CTZ (Figures 2 and 3). In marked contrast, both γ-5 and γ-7 selectively enhanced the maximal glutamate-evoked current of GluR2 in the presence of CTZ with minimal effects on GluR1, GluR3, and GluR4. Collectively, the preceding data in the absence and presence of CTZ demonstrate that, in contrast to the augmentation of all GluRs by the type I TARPs, including stargazin (Kott et al., 2007), γ-5 and γ-7 selectively enhance responses of GluR2 and GluR1-2, respectively. This characteristic and others described below distinguish γ-2, γ-3, γ-4, and γ-8 from γ-5 and γ-7; therefore, we will hereafter refer to these families as type I TARPs and type II TARPs, respectively.

As GluR1 coimmunoprecipitated with γ-5 from cerebellum (Figure 1A), we explored functional modulation of GluR1 by γ-5. Recent studies showed that type I TARPs modulate the rectification of AMPA receptor-mediated currents (Kott et al., 2008; Soto et al., 2007). Similar to type I TARPs, γ-5 (and γ-7) also modulated the rectification of GluR1 currents (Figure S2), implying that γ-5 physically associates with GluR1. Our functional data suggest that type II TARPs also bind to GluR3 homomers, as γ-5 and γ-7 reduced the peak currents of GluR3, and γ-7 increased steady-state currents of GluR3 (Figure 3). These observations indicate that type II TARPs associate broadly with GluRs, but that regulation is strictly subunit specific.

## The Q/R Site in GluR2 Determines Subunit-Selective Gating of Type II TARPs

The differential effects of γ-5 on gating of GluR2 versus other subunits suggested a possible role for the "Q/R site" in the pore region of the channel, which undergoes mRNA editing only in GluR2 to produce an Arg residue that regulates channel rectification and ion permeability (Sommer et al., 1991). To determine the contribution of this site to type II TARP modulation of GluR2, we mutated this residue in GluR2 to Gln (Q). Strikingly, we found that the enhancing effects of γ-5 on both the glutamate-evoked peak current and CTZ-enhanced maximal current were eliminated in GluR2Q (Figure 3). In addition, γ-7 enhanced only the steady-state currents of GluR2Q, and γ-7 had no effect on peak currents in the absence or presence of CTZ. In contrast, γ-2 modulation of glutamate-evoked peak, steady-state, and CTZ-enhanced currents was unaffected by the Gln mutation (Figure 3). To evaluate further the dependence of γ-5 effects on Q/R editing, we constructed an unnatural R-form of the flip splice variant of GluR1. Similar to its effects on GluR2iR, γ-5 enhanced glutamate-evoked peak and steady-state currents through GluR1iR, as well as the currents evoked by glutamate in the presence of CTZ. Furthermore, γ-5 reduced by 7-fold the potency of glutamate for GluR1iR (Figure S3). Quantitatively, all TARPs tested here showed larger increases of GluR2R-mediated currents as compared to GluR2Q receptors (Figure 3). This preferential effect of TARPs on R-forms is also observed in GluR1iR (Figure S3). As virtually all GluR2 subunits, but no other AMPA receptor subunits are edited at the Q/R site (Sommer et al., 1991), these data suggest that only neuronal receptors containing the GluR2 subunit would be enhanced by γ-5.

## γ-5 Accelerates GluR2 Channel Deactivation and Desensitization

The preceding results demonstrate that γ-5 uniquely modulates GluR2 by enhancing peak currents and suppressing steady-state responses. A hallmark of type I TARPs is their ability to slow AMPA receptor gating by modulating the deactivation and/or desensitization processes of these ion channels. To determine the extent to which γ-5 modulates these biophysical processes, we compared the effects of coexpression of γ-2 or γ-5 on deactivation and desensitization of GluR2. To evaluate

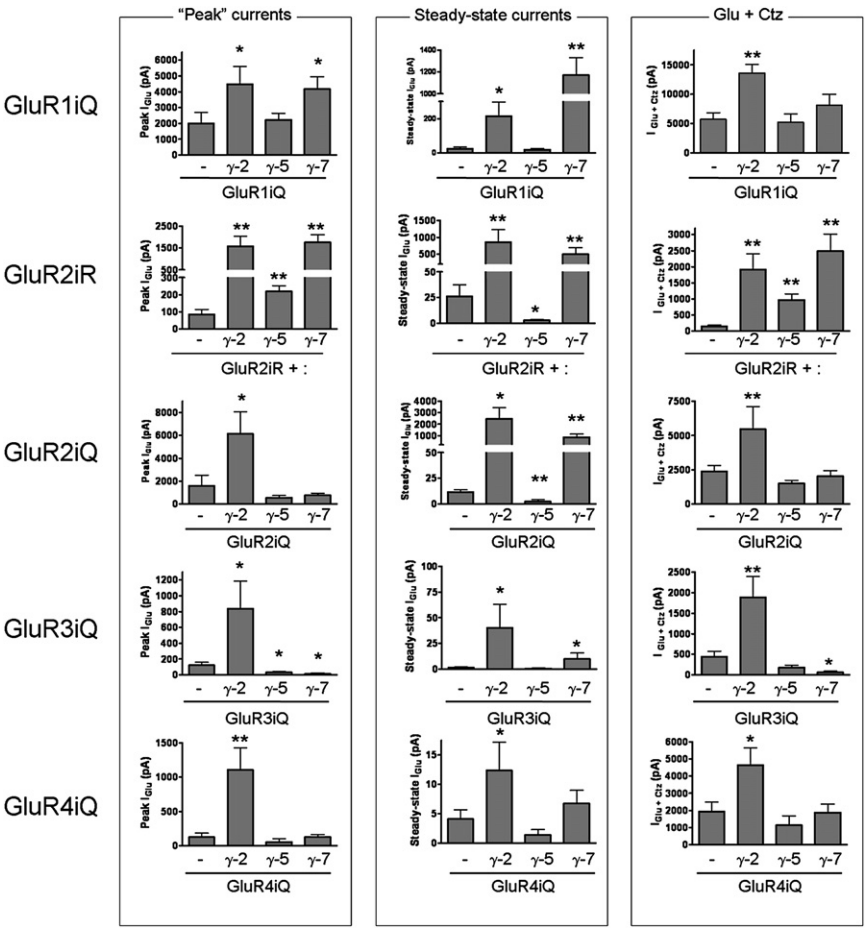


**Figure 3. Subunit-Specific Modulation of GluR Homomers by Type II TARPs**

Peak and steady-state currents were recorded from HEK293T cells transfected with GluR1iQ, GluR2iR, GluR2iQ, GluR3iQ, or GluR4iQ and γ-2, γ-5, or γ-7 in response to application of glutamate (1 mM) alone (left and middle panels) or in the presence of CTZ (40 μM) (right panel). γ-5 regulates GluR2iR robustly but has minimal effects on other AMPA receptor subunits. γ-5 does not enhance the maximal currents evoked by glutamate in the presence of CTZ from the "unedited" GluR2iQ, indicating that Q/R editing of GluR2 is critical for regulation by γ-5. γ-7 robustly amplifies currents from GluR1iQ, GluR2iQ, and GluR2iR, but not GluR3iQ or GluR4iQ. In contrast, γ-2 regulates all AMPAR subunits. Error bars, SEM. Statistical significance with respect to no TARPs (Student's t test): *$p < 0.05$, **$p < 0.01$.

the effects of these two TARPs on deactivation, we used ultrafast perfusion of 1 ms pulses of glutamate to outside-out patches (Figure 4A). Because GluR2 subunits show very small channel conductances (Swanson et al., 1997), we could not detect glutamate-evoked currents in patches from cells transfected with GluR2R alone (data not shown). However, cotransfection with GluR2R with either γ-5 or γ-2 yielded readily detectable currents. Results showed that the deactivation rates of these currents were significantly faster in cells transfected with γ-5 as compared to γ-2 (Figures 4A and 4C).

To evaluate desensitization we used ultrafast application of glutamate to isolated cells, which allowed detection of currents from GluR2iR transfectants. We found that γ-2 slowed the desensitization of GluR2iR (Figures 4B and 4D), but to a lesser extent than seen previously with GluR1i (Tomita et al., 2005). By contrast, γ-5 accelerated the desensitization rate of GluR2iR currents (Figures 4B and 4D). Furthermore, γ-2 enhanced steady-state currents from GluR2i(R), whereas γ-5 nearly abolished these currents (Figure 4E).

In an attempt to confirm that endogenously expressed γ-5 can influence the function of native AMPA receptors, we took advantage of the selective expression of γ-5 in the CA2, but not the CA1 or CA3 pyramidal cells of the hippocampus (Figure 4F; Fukaya et al., 2005; Lein et al., 2007). We assessed AMPA receptor-mediated desensitization in acutely isolated CA2 and CA3 hippocampal pyramidal neuron using ultrafast glutamate application. Results showed that the rate of desensitization of AMPA-receptor-mediated currents from CA2 neurons was significantly faster than that from CA3 neurons (Figures 4G and 4H). In addition the extent of desensitization was greater in CA2 neurons than CA3 neurons, as revealed by a lower steady-state to peak current ratio (Figures 4G and 4I). Taken together, these data support the hypothesis that γ-5 can uniquely modulate the biophysical properties of native neuronal AMPA receptors.

## γ-5 Decreases Agonist Affinity for GluR2 Subunits

Previous studies showed that type I TARPs increase the affinity of GluR1 receptors for glutamate, whereas γ-7 does not alter agonist affinity (Kato et al., 2007). Here, similar to the effects of γ-7, the present results showed that γ-5 had no effect on glutamate affinity for GluR1 (Figure 5). However, for GluR2, γ-5 *lowered* the glutamate affinity by ~10-fold, and this effect was restricted to the edited form of GluR2(R) (Figure 5). Evaluation of GluR1/2 heteromers revealed that γ-5 had an intermediate effect, producing ~3-fold rightward shift in glutamate affinity. To confirm that our GluR1 and GluR2 cotransfectants formed heteromeric receptors, we assessed channel rectification. As shown in Figure S4, GluR1/2 and GluR2/3 cotransfectants showed similar rectification indices to GluR2 homomers, which is consistent with the dominant influence of GluR2 on heteromeric receptor rectification (Hollmann et al., 1991; Sommer et al., 1991).

## γ-5 Does Not Enhance GluR Subunit Surface Trafficking

Previous work has shown that γ-2 enhances surface expression of AMPA receptors in both neurons and nonneuronal cells (Tomita et al., 2005). In COS cells, trafficking by γ-2 can be quantified by staining transfected cells for an extracellular epitope on

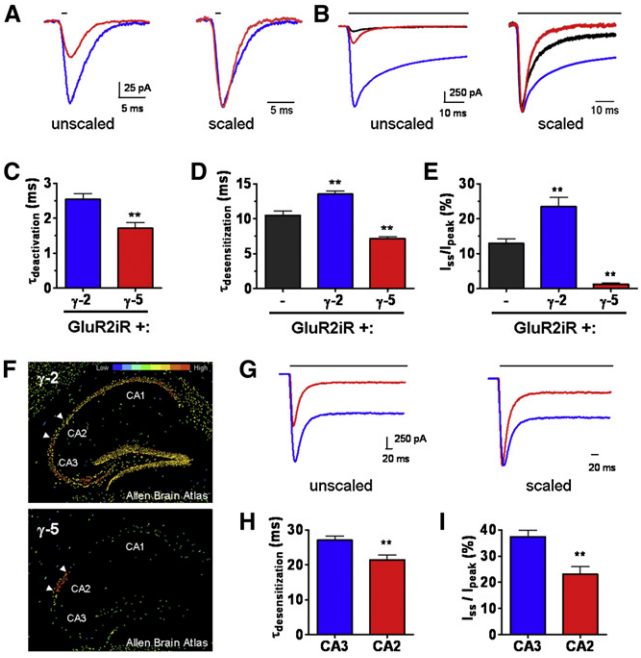


**Figure 4. γ-5 Accelerates Deactivation and Both Accelerates and Enhances the Extent of Desensitization of GluR2iR-Containing AMPA Receptors**

(A–E) HEK293T cells were transfected with GluR2iR alone or GluR2iR with γ-2 or γ-5. The kinetics of deactivation and desensitization and the ratio of steady-state to peak currents were quantified. (A and B) Typical traces of deactivation ([A] left, unscaled; right, scaled) and desensitization (B). Bars show glutamate application. Black, GluR2iR alone; blue, GluR2iR + γ-2; red, GluR2iR + γ-5. (C) Outside-out patches from the transfected cells held at −80 mV were perfused with glutamate for 1 ms. GluR2iR + γ-5 shows faster deactivation kinetics than GluR2iR + γ-2. The traces were fit with a single exponential function. Glutamate-evoked currents from patches of GluR2iR without TARPs were not detectable (data not shown). (D) γ-5 accelerates and γ-2 decelerates the rate of GluR2iR desensitization. Desensitization traces were fit using a single exponential function. (E) γ-5 reduces and γ-2 increases the ratio of steady-state to peak current amplitude. Error bars, SEM. Statistical significance with respect to γ-2 (C) and no TARPs (D and E), respectively (Student's t test [C], Fisher's LSD [D and E]).

(F) In situ hybridization from the Allen Brain Atlas (Lein et al., 2007) shows broad expression of γ-2 in hippocampal pyramidal neurons and selective localization of γ-5 in CA2 (arrowheads) pyramidal neurons.

(G) Typical traces of AMPA receptor-mediated currents evoked by glutamate in acutely isolated CA2 (red) and CA3 (blue) hippocampal pyramidal neurons. Glutamate was perfused using a piezoelectric actuator.

(H and I) Desensitization occurred faster (H) and to a greater extent (I) in CA2 as compared to CA3 pyramidal neurons.

GluR1 both before and after plasma membrane permeabilization (Vandenberghe et al., 2005a). Using this assay, we found in the present studies that γ-2 enhanced the cellular trafficking of GluR1 and GluR2. By contrast, γ-5 did not augment the cellular trafficking of either GluR1 or GluR2 (Figures 6A and 6B). As a second method to quantify surface AMPA receptors, we employed a membrane-impermeable divalent crosslinker, bis[sulfosuccinimidyl]suberate ($BS^3$), which selectively crosslinks surface proteins (Hall and Soderling, 1997). γ-2, but not γ-5, selectively increased crosslinked AMPA receptors (Figure 6C). Collectively, these data indicate that the influence of γ-5 on AMPA receptors is restricted to effects on ligand binding and channel gating.

## Type II TARPs Differentially Regulate Heteromeric AMPA Receptors

Many neuronal AMPA receptors comprise heterotetramers. Previous studies have indicated that GluR1/2 heteromeric receptors represent the primary AMPA receptor type in hippocampal neurons, whereas GluR2/3 heteromeric receptors represent a prominent subunit combination in principal neurons of cerebral cortex (Geiger et al., 1995). In the present studies, coexpression of either γ-2 or γ-7 with GluR1/2 robustly augmented the peak and steady-state currents (with or without CTZ) from these receptors compared to either GluR1 or GluR2 alone, whereas γ-5 was without effect on GluR1/2 heteroreceptors (Figure 7). In contrast, all three TARPs enhanced glutamate-evoked currents from GluR2/3-expressing cells (Figure 7).

Several previous studies of TARPs have focused on rescue experiments in stargazer cerebellar granule cells (Chen et al., 2000; Kato et al., 2007; Tomita et al., 2003), which predominately express flop isoforms of GluR2 and GluR4 (Monyer et al., 1991). These studies showed that type I TARPs fully restored functional AMPA receptors, whereas γ-7 only partially rescued functional AMPA receptors and γ-5 had no effect (Kato et al., 2007). We modeled these cerebellar granule cell experiments by cotransfecting the flop isoforms of GluR2 and GluR4 with γ-2, γ-5, or γ-7 and then assessed currents evoked by glutamate in the presence of CTZ. Mimicking the results reported previously from stargazer granule cells (Kato et al., 2007), γ-2 robustly enhanced currents, γ-7 had a lesser effect, and γ-5 was inactive (Figure S5).

## γ-5 Modulates Neuronal AMPA Receptors

Our experiments described above using acutely isolated CA2 and CA3 neurons indicated that endogenous γ-5 can influence channel gating of neuronal AMPA receptors. To assess further the effects of γ-5 in neuronal receptors, we transfected this TARP into cerebrocortical neurons, which normally express significant GluR2/3 heteromers. Cultured neurons from rat embryos were transfected on DIV 3 and recorded DIV 8. To evaluate deactivation and desensitization kinetics, we measured AMPA receptor-mediated currents from pulled outside-out patches held at −80 mV and perfused with a glutamate solution containing $GABA_A$ and NMDA receptor blockers. In outside-out patches from these neurons, we found that—similar to its effects on GluR2—γ-5 transfection in cerebrocortical neurons increased the rate and dramatically enhanced the extent of AMPA receptor desensitization (Figures 8A–8C). Note that outside patches have a much smaller surface area and can be efficiently perfused with an ultrafast system; therefore, steady-state/peak current ratios in Figures 8A and 8C were significantly smaller than those in Figure 4I, which used whole-cell recordings. In these experiments, currents recorded from cells cotransfected with γ-5 showed a trend toward faster rates of deactivation, but this effect was not significant (Figures 8D and 8E). In addition, a concentration-response analysis showed that γ-5 reduced glutamate potency by 2-fold (Figure 8F).

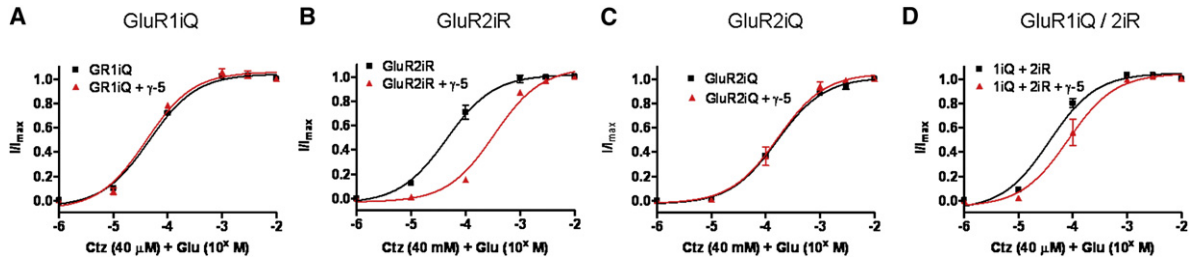


**Figure 5. γ-5 Lowers the Affinity of Glutamate on GluR2R-Containing AMPA Receptors**

(A–D) Concentration-response profiles for glutamate (in the presence of 40 μM CTZ) measured from HEK293T cells expressing GluRs either alone or with γ-5. Coexpression with γ-5 decreased the potency of glutamate for GluR2iR homomer and GluR1iQ/GluR2iR heteromer, but not from GluR1iQ homomer. Also, γ-5 did not change the potency of GluR2iQ, indicating that the γ-5 effect on glutamate potency requires mRNA editing.

## DISCUSSION

### γ-5 and γ-7 Represent a Distinct Family of TARPs

These studies demonstrate multiple effects of γ-5 on AMPA receptors that differ dramatically from the actions of the originally identified TARPs, and thereby identify γ-5 as a distinct TARP isoform. First, γ-5 shows specificity to enhance glutamate-evoked currents from receptors containing the R-edited form of GluR2. Second, γ-5 markedly *lowers* the affinity of certain AMPA receptors for glutamate. Third, γ-5 enhances maximal currents of GluR2 subunits while simultaneously reducing steady-state currents. Finally, γ-5 mediates all these channel effects without promoting the surface expression of GluR2. As γ-7 shows extremely high sequence homology with γ-5 and shares some of these unique regulatory properties, we now define these two proteins as type II TARPs.

While surprising, these data are entirely consistent with our previously published data on γ-5 and γ-7. Several studies have shown that γ-5 does not promote surface trafficking or glutamate-evoked currents from GluR1 homomers (Tomita et al., 2003). In addition, previous studies have demonstrated that γ-5 does not augment glutamate-evoked currents in stargazer granule cells, which primarily express GluR2 and GluR4 flop isoforms (Tomita et al., 2003). Furthermore, γ-7 only modestly enhances maximal glutamate-evoked currents in transfected stargazer granule cells (Kato et al., 2007). As these studies with stargazer granule cells transfected with γ-7 used glutamate in the presence of CTZ, they are consistent with the present results showing that γ-7 only moderately augments currents from GluR2/GluR4 flop heteromers treated with Glu and CTZ. Finally, previous work has shown that a chimeric construct of stargazin containing the extracellular domain of γ-5 accelerates the decay kinetics of EPSCs in transfected hippocampal neurons (Tomita et al., 2005) consistent with the ability of γ-5 to accelerate receptor deactivation.

### γ-5 Controls Only Channel Gating, Not Receptor Trafficking

The original studies implied that stargazin functions solely by promoting AMPA receptor trafficking (Chen et al., 2000). Subsequent experiments showed that stargazin and all type I TARPs control both AMPA receptor trafficking and gating (Bedoukian et al., 2006; Priel et al., 2005; Tomita et al., 2005; Turetsky et al., 2005). In marked contrast to the effects of type I TARPS, the present results indicate that γ-5 functions only to regulate AMPA receptor gating. As a consequence, the mechanisms underlying the enhancement of maximal currents from GluR2 by γ-5 likely involve the augmentation of single-channel conductance and/or burst times as have been reported for stargazin (Tomita et al.,

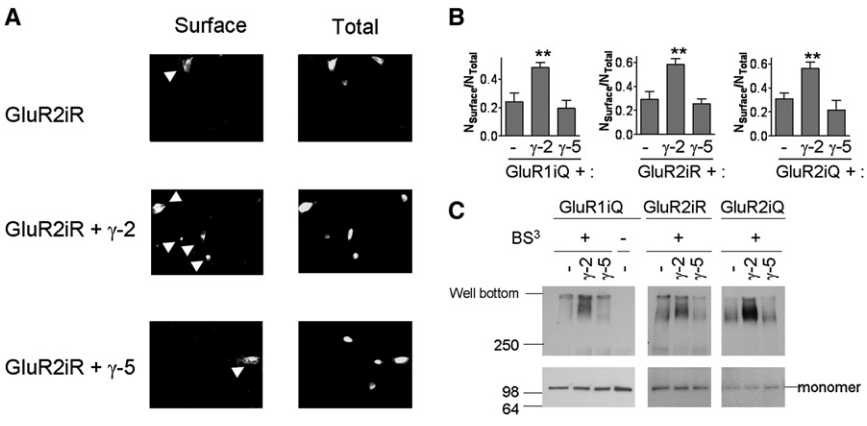


**Figure 6. γ-5 Effects on GluR2-Containing AMPA Receptors Do Not Involve Cell Surface Protein Trafficking**

(A and B) CHO cells coexpressing HA-tagged GluR1iQ or untagged GluR2iR/2iQ with γ-2 or γ-5 were labeled with an antibody to the HA-tag or to the ectodomain of GluR2 without permeabilization. After fixation and permeabilization, the cells were incubated with an antibody to the cytoplasmic domain of GluR1 or GluR2 as indicated. (A) Typical images of surface and total staining of GluR2 either alone or coexpressed with γ-2/5. (B) The ratios of surface to total GluR1/2-positive cell were quantified by an observer blinded to the transfection condition. Error bars, SEM. Statistical significance with respect to no TARPs (Student's t test): $^{**}p < 0.01$.

(C) CHO cells expressing GluRs and γ-subunits were treated with a membrane-impermeable crosslinker, $BS^3$. After washing the cells with buffer containing primary amine, proteins were separated by SDS-PAGE and blotted with anti-GluR1 or anti-GluR2/3 antibodies. Intracellular GluRs were detected as monomers at ~100 kD, whereas surface GluRs migrated at higher molecular weight. γ-2 but not γ-5 increased crosslinked GluRs, indicating that the surface trafficking of GluRs was promoted by γ-2 but not by γ-5.

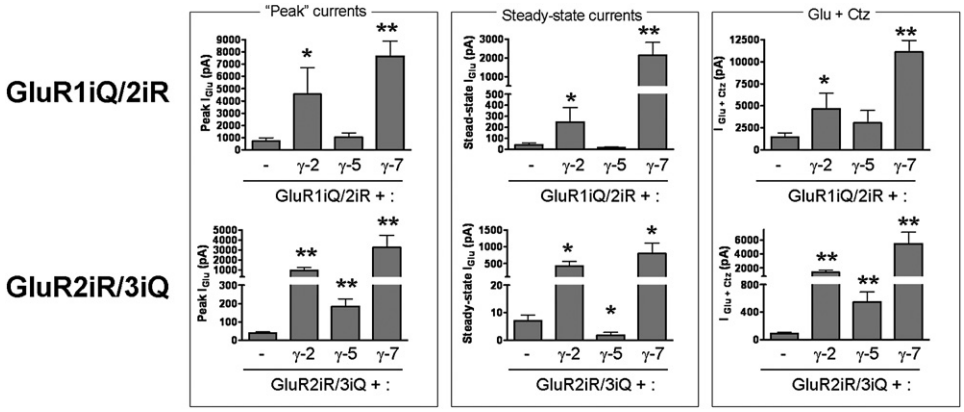


**Figure 7. γ-5 Preferentially Regulates AMPA Receptor Heteromers Composed of GluR2/3 Subunits**

Peak and steady-state currents were recorded from HEK293T cells transfected with GluR1iQ/GluR2iR or GluR2iR/GluR3iQ and γ-2, γ-5, or γ-7 in response to application of glutamate (1 mM) alone (left and middle panels) or in the presence of CTZ (40 μM) (right panel). These GluR combinations were chosen to represent cortical and hippocampal neurons, which mainly express GluR1iQ/GluR2iR and GluR2iR/GluR3iQ, respectively. γ-5 enhanced peak currents to glutamate alone and maximal currents in the presence of CTZ from GluR2iR/GluR3iQ but not from GluR1iQ/GluR2iR. In contrast, γ-2 and γ-7 regulated both heteromers in a similar manner. Error bars, SEM. Statistical significance with respect to no heteromers in the absence of TARPs (Student's t test): *p < 0.05, **p < 0.01.

2005). The inability of γ-5 to promote surface expression of AMPA receptors resembles a *C. elegans* stargazin-like molecule, which reconstitutes functional invertebrate glutamate-gated channels without a role in receptor trafficking (Walker et al., 2006). It is interesting to speculate that perhaps type II TARPs initially derived from their *C. elegans* orthologs, and later type I TARPs evolved capacity for AMPA receptor trafficking.

## Type II TARPs Have Unique Effects on Glutamate Affinity and Channel Gating

Previous studies have shown that all type I TARPs increase affinity of glutamate for AMPA receptors and as a consequence slow the deactivation process. In addition, type I TARPs all decelerate the rate of AMPA receptor desensitization and attenuate the extent of receptor desensitization (Bedoukian et al., 2006; Priel et al., 2005; Tomita et al., 2005; Turetsky et al., 2005). In striking contrast, the present studies show that γ-5 reduces the affinity of glutamate for GluR2-containing AMPA receptors and as a result accelerates receptor deactivation. In addition, γ-5 increases the both the rate and extent of AMPA receptor desensitization such that the steady-state currents of GluR2-containing receptors are nearly abolished. Previous studies have shown that γ-7 also has distinct effects from type I TARPs in that it does not alter the affinity of AMPA receptors for glutamate and has only minimal effects on the rate of receptor deactivation (Kato et al., 2007). Intriguingly, γ-7 imparts an unusual effect on the desensitization process of GluR1 and GluR2 receptors marked by an initial decay in current from the peak response followed by a slow increase in current to steady state.

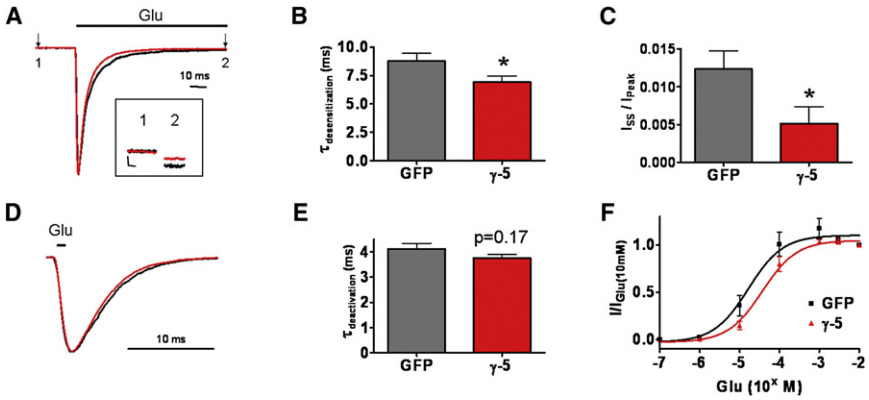


**Figure 8. Transfection of γ-5 into Neuronal AMPA Receptors Modifies Their Gating Properties**

Dissociated cultures from rat cerebral cortices were transfected with either γ-5 and GFP or GFP alone.

(A) Typical traces recorded from the outside-out patches evoked by 100–400 ms application of 100 mM glutamate. Inset shows the currents recorded at the times indicated by the arrows 1 and 2. Black and red traces represent recordings from cells expressing GFP alone and γ-5 plus GFP, respectively.

(B) γ-5 (n = 10) significantly accelerated desensitization of neuronal AMPA receptors (n = 9).

(C) Steady-state currents were recorded before and 90–390 ms after the application of glutamate. γ-5 (n = 10) reduced steady-state currents of AMPA receptors (n = 9).

(D) Typical traces recorded from outside-out patches evoked by 1 ms application of 100 mM glutamate. Black, GFP alone; red, γ-5 plus GFP.

(E) Coexpression of cortical neurons with γ-5 produced a tendency toward an increase in the rate of deactivation.

(F) Concentration-response profiles for glutamate (in the presence of 40 μM CTZ) measured from cultured neurons with and without γ-5. Results showed that transfection of γ-5 into cortical neurons reduced the potency of glutamate at the receptor. Error bars, SEM. Statistical significance with respect to recordings in the absence of γ-5 coexpression (Student's t test): *p < 0.05.

## Subunit-Specific AMPA Receptor Regulation by Type II TARPs

Whereas type I TARPs can potentiate all AMPA receptor combinations (Kott et al., 2007), type II TARPs display exquisite subunit-specific regulation. γ-7 enhances peak currents only from channels containing GluR1 or GluR2. γ-5 displays even stricter specificity in that it augments currents only from GluR2-containing AMPA receptors for which a single amino acid in the pore region has been edited from a glutamine to an arginine residue. The arginine in edited forms of GluR2 confers low calcium permeability (Sommer et al., 1991), low single-channel conductance (Swanson et al., 1997), and an approximately linear current-voltage relation even in heteromeric receptors (Verdoorn et al., 1991). This mRNA editing of GluR2 is critical for normal development and survival, as mice engineered with an editing deficiency in GluR2 display severe neurological impairment and premature death (Brusa et al., 1995). The importance of Q/R site editing of GluR2 notwithstanding, the present results indicate that the peak monovalent cation current through either homomeric GluR2R receptors or GluR2R/GluR3 receptors can be enhanced by coexpression with multiple TARPs. However, unlike the effects of other TARPs, γ-5 would be expected to rapidly terminate current through these receptor subtypes through acceleration of the deactivation and desensitization. Given the heterogeneous distribution of TARPs in the CNS, these data suggest that the permeability of GluR2-containing AMPA receptors can be fine-tuned in specific brain regions.

The mechanistic basis for this subunit specific specificity remains unclear. Previous work showed that TARPs interact with both the extracellular glutamate binding module and the transmembrane domains of AMPA receptors (Tomita et al., 2004, 2007). The selective regulation of edited GluR2 by γ-5 suggests that TARPs may also interact with the ion channel pore. Interestingly, recent studies have shown that stargazin regulation of GluR1 depends critically upon this same Q/R residue (Korber et al., 2007a). Furthermore, stargazin and other type I TARPs attenuate intracellular polyamine block of calcium permeable AMPA receptors, which is dictated by the Q residue in non-GluR2 subunits (Soto et al., 2007). Whether TARPs directly bind to or allosterically regulate this constricted region of the AMPA receptor pore will require structural studies.

## Roles for Type II TARPs in AMPA Receptor Physiology and Plasticity

The present data have important implications for AMPA receptor physiology in synaptic transmission and plasticity. Though our antibody was unsuitable for immunohistochemistry, in situ hybridization as part of the Allen Brain Atlas (Lein et al., 2007) shows that γ-5 mRNA is enriched in Bergman glia, as well as a variety of neuronal populations including locus coeruleus, olfactory bulb, lateral septal nucleus, interpeduncular nucleus, and the CA2 and rostral/medial CA1 regions of hippocampus (Figure S6). The regulatory features of γ-5 may underlie unique properties of AMPA receptors in these cell types. Previous studies indicate that AMPA receptors in Bergmann glia show very rapid channel gating, consistent with the regulatory properties conferred by γ-5. Likewise, the present studies show that hippocampal CA2 neurons that express γ-5 display more rapid and more complete AMPA receptor desensitization than CA3 neurons which do not contain this TARP. The extent to which γ-5 expression confers a unique phenotype in AMPA receptor gating in other cell types that express multiple TARPS will require future studies.

The subunit-specific regulation of AMPA receptors by type II TARPs has important physiological implications. Previous work has suggested that subunit-specific rules govern the trafficking and plasticity of neuronal AMPA receptors. Specifically, receptors containing long C-terminal tailed GluR1 are suggested to traffic in a regulated fashion to mediate long term potentiation, whereas receptors containing only short-tailed GluR2/3 are thought to traffic constitutively and mediate long-term depression (Lee et al., 2004; Malinow and Malenka, 2002; Song and Huganir, 2002). PDZ domain interactions with the distinct C termini of short- and long-tailed GluR subunits have been suggested to underlie these processes; however, certain discrepancies have arisen (Kim et al., 2005; Meng et al., 2003). The subunit-specific regulation of GluR subunits by type II TARPs may also contribute to synaptic plasticity.

As AMPA receptors require a TARP to traffic to the neuronal cell surface, type II TARPs may cooperate with their type I homologs to regulate AMPA receptors expression and function. Several possible mechanisms could underlie such collaboration. The number of TARPs associated with a tetrameric AMPA receptor remains uncertain; however, recent work suggests that this stoichiometry can vary (Milstein et al., 2007). We previously detected some γ-2 in our γ-7 immunoprecipitates from cerebellum, suggesting that these proteins may occur together in neuronal AMPA receptors (Kato et al., 2007). Our other studies show that glutamate can cause dissociation of type I TARPs from neuronal AMPA receptors, and this could conceivably lead to an exchange in TARP isoform regulation once the receptors have been delivered to the cell membrane (Tomita et al., 2004). Defining quantitatively TARP stoichiometries and the dynamics of their interactions with AMPA receptors remain crucial issues. Future studies of mice lacking type II TARPs should help elucidate the role for these proteins in synaptic transmission and plasticity.

## EXPERIMENTAL PROCEDURES

### Antibodies

The following commercially acquired antibodies were used: rabbit polyclonals to C-terminal GluR1 (AB1504; Millipore), C-terminal GluR2/3 (AB1506; Millipore), and C-terminal GluR4 (AB1508; Millipore); and mouse monoclonals to N-terminal GluR2 (MAB397; Millipore) and HA.11 (16B12; Covance). Polyclonal antiserum to γ-5 was described (Kato et al., 2007).

### Subcellular Fractionation

Subcellular fractions were prepared by differential centrifugation as described (Kato et al., 2007). Briefly, ten rat olfactory bulbs were homogenized in 10 ml of buffer I (0.32 M sucrose, 3 mM HEPES-Na [pH 7.4], 0.1 mg/ml PMSF). The homogenate was centrifuged at 1000 × g to produce a pellet (P1) and a supernatant (S1). P1 pellet was resuspended in buffer I and centrifuged at 1000 × g to obtain crude nuclear fraction (P1′) and a supernatant (S1′). The combined supernatant (S1 + S1′) was centrifuged at 12,000 × g for 15 min to produce a pellet (P2) and supernatant (S2). The S2 fraction was centrifuged at 33,000 × g for 20 min to give a pellet (P3) and a supernatant (S3). The S3 fraction was centrifuged at 260,000 × g for 2 hr to produce a pellet (P4) and a supernatant (S4, cytosolic fraction). The P2 pellet was resuspended in the original volume of buffer I and centrifuged for 15 min at 13,000 × g to yield the crude synaptosomal fraction (P2′). The P2′ fraction was resuspended in homogenization buffer and

hypotonically lysed by addition of 9 vol of ice-cold water with 4 mM HEPES (pH 7.4) and incubated for 1 hr. The lysate was centrifuged for 20 min at 33,000 × g to produce the lysate heavy membrane pellet (LP1) and lysate supernatant (LS1). The LS1 fraction was then centrifuged for 20 min at 260,000 × g for 2 hr to give a crude synaptosomal vesicle pellet (LP2) and a cytosolic synaptosomal supernatant (LS2). In a separate procedure, synaptosomes and PSD fractions were prepared from ten rat cerebella. The synaptosome fraction was purified by discontinuous sucrose density gradient centrifugation was extracted once or twice with ice-cold 0.5% Triton X-100 in 6 mM Tris-HCl (pH 7.5) and then centrifuged to obtain the PSD I and PSD II pellets.

### Immunoprecipitation

Immunoprecipitation was performed as described (Kato et al., 2007). Briefly, rat cerebella were homogenized with 3-fold tissue volume of buffer I (0.32 M sucrose, 3 mM HEPES-Na [pH 7.4], 0.1 mg/ml PMSF) and spun at 20,000 × g for 10 m at 4°C. The resulting pellets were homogenized with 4-fold the original volume of buffer I and then solubilized with 0.1% Triton X-100 for 1 hr. The solubilized extracts after centrifugation at 100,000 × g were precleared by the addition of protein A-Sepharose (GE Healthcare). This supernatant was incubated with appropriate antibodies (20 μg) for 1 hr and then with 25 μl of protein A-Sepharose for 1 hr. The resultant resin was washed eight times with buffer I containing 20 mM NaCl. Adherent proteins were eluted with Laemmli sample buffer with 3% SDS at 55°C for 30 min and then 95°C for 10 min and separated by SDS-PAGE. The separated proteins were transferred onto PVDF membrane and blotted with polyclonal antibodies to GluR1 (1:400), GluR2/3 (AB1506, 1:400), GluR4 (1:400), and γ-5 (1:100).

### Cell Culture

Primary cultures of rat cortical neurons were prepared essentially as described (Kato et al., 2001). Briefly, embryonic Wistar rats (gestational day 19) were removed from euthanized mothers, and the brains were dissected. The cortices were incubated at 37°C for 10 min in a papain solution: 5 mM L-cysteine, 1 mM EDTA, 10 mM HEPES-NaOH (pH 7.4), 100 μg/ml bovine serum albumin, 10 unit/ml papain (Worthington) and 0.02% DNase (Sigma). The reaction was stopped by addition of an equal volume of fetal bovine serum. The cells were gently triturated and washed with the plating medium, Neurobasal (Invitrogen) supplemented with B-27, 100 μg/ml penicillin, 85 μg/ml streptomycin, 0.5 mM glutamine, and 10 μM 2-mercaptoethanol. The cells were plated on the 12 mm coverslips coated with poly-D-lysine in 24-well stacks at 100,000 cells/well density. Fresh plating medium without 2-mercaptoethanol was added 2–3 days after plating (2 ml medium total/well).

A γ-5 CMV expression vector (0.1 μg/well) was mixed with a GFP-expressing plasmid (0.1 μg/well), and DNA-Lipofectamine 2000 (Invitrogen) complexes were prepared in Neurobasal medium. Primary neurons (4–5 days in vitro [DIV]) were incubated with these Lipofectamine complexes for 2–5 hr, and the original culture medium was retained. After transfection, the cells were washed twice, and then the conditioned medium was replaced. Electrophysiological recordings from primary neurons were carried out at 7–9 DIV, which is 3–4 days after transfection.

Exogenous expression of γ-5 in primary neurons using a Semliki forest viral vector was performed as described (Kato et al., 2005). Briefly, γ-5 cDNA was subcloned into pSCA1, and viral particles were produced by transfecting pSCA1-γ-5 and pHelper into HEK293T cells. Supernatants were harvested 48 hr after infection. Prior to infection, the viral particles were activated with chymotrypsin. Viral solution was added to the cultures (3 weeks in vitro), and the cells were stained 24–48 hr after infection. The cells were fixed and permeabilized with 100% methanol on ice. After blocking with 7.5% BSA, the rabbit anti-γ-5 (1 μg/μl) and the mouse anti-PSD-95 (1:100) antibodies were overlaid onto the cells. The primary antibodies were visualized with Alexa 488 conjugated anti-rabbit IgG and Alexa564 conjugated anti-mouse IgG (Invitrogen).

### Electrophysiology

Glutamate-evoked currents were recorded from primary cultures of rat cortical neurons or HEK293T cells transiently transfected with specific GluR subunits and TARPs using previously described procedures (Kato et al., 2007). Recording electrodes were pulled from borosilicate capillary tubing (Corning 7052; World Precision Instruments, Sarasota, FL) using a multistage puller (model P-97; Sutter Instruments, Novato CA). The electrodes were fire polished using a microforge (model MF-830; Narishige, Tokyo, Japan) before use. For experiments using the whole-cell variant of the patch-clamp technique, the internal electrode solution contained the following: 130 mM CsCl, 2.5 mM $MgCl_2$, 0.5 mM $CaCl_2$, 1 mM EGTA, 10 mM HEPES, 4 mM MgATP, 12 mM phosphocreatine (pH 7.20 ± 0.03), 280 mOsm adjusted with phosphocreatine. For experiments measuring the current-voltage relationship of AMPA receptors, 100 μM spermine was added to this internal solution. The extracellular solution contained: 135 mM NaCl, 5.4 mM KCl, 1.0 mM $MgCl_2$, 1.8 mM $CaCl_2$, 5 mM HEPES (pH 7.2), 300 mOsm.

Before recording, transfected HEK cells were placed into a 50 mm transparent plastic Petri dish that was mounted onto the stage of an inverted microscope. After the recording electrode was placed in the extracellular solution bath, offset potentials were corrected, and electrode resistances ranged between 2 and 7 MΩ. Voltage-clamp recordings were made using an Axopatch 200B amplifier (Medical Devices Inc, CITY, CA), and currents were digitized and monitored with pClamp software version 10.2 (Medical Devices Inc., CITY, CA) running on a personal computer. The membrane potential of cells was held at −80 mV unless stated otherwise. A small amount of constant positive pressure (2–3 $cmH_2O$) was applied to the electrodes as they were advanced through the bath. After achieving the whole-cell configuration, series resistance was compensated (70%–85%) and monitored periodically. Recordings were made from single green fluorescent protein (GFP)-positive cells at room temperature.

Hippocampal CA2 and CA3 neurons from young male Sprague-Dawley rats (12–16 days old) were acutely isolated using procedures described previously (Baumbarger et al., 2001). Brains were removed rapidly and immersed in a cold (~2°C) $NaHCO_3$-buffered saline solution containing 120 mM NaCl, 2.5 mM KCl, 1 mM $MgCl_2$, 1.25 mM $Na_2PO_4$, 2 mM $CaCl_2$, 26 mM $NaHCO_3$, and 10 mM glucose (pH 7.4), osmolarity 300 ± 5 mOsM/l. The brains were blocked, and 400 μm thick coronal sections were cut using a Vibroslice (Campden Instruments, London, England). Slices were then incubated at room temperature in a holding chamber containing the continuously oxygenated (95% $O_2$, 5% $CO_2$) $NaHCO_3$-buffered saline solution and allowed to recover for at least 30 min. Slices were transferred to a Petri dish containing a low-$Ca^2$ HEPES-buffered saline solution containing 140 mM $NaHOCH_2CH_2SO_3$ (Na isethionate), 2 mM KCl, 4 mM $MgCl_2$, 0.1 mM $CaCl_2$, and 15 mM HEPES (pH 7.4), osmolarity 300 ± 5 mOsM/l adjusted with glucose, and placed under a dissecting microscope. The CA2 or CA3 regions were dissected out from the surrounding tissue and placed into a holding chamber containing protease type XIV (1 mg/ml; Sigma-Aldrich, St. Louis, MO) dissolved in oxygenated HEPES-buffered Hank's balanced salt solution (HBSS 6136; Sigma-Aldrich) maintained at 37°C, pH 7.4, osmolarity 300 ± 5 mOsM/l. After 30 min of incubation in the enzyme solution, the tissue was rinsed three times with the low-$Ca^{2+}$ HEPES-buffered saline solution and triturated using three fire-polished Pasteur pipettes having tips of decreasing diameter. Before whole-cell recording, the cell suspension was placed into a 50 mm transparent plastic Petri dish that was mounted onto the stage of an inverted microscope. CA2 and CA3 pyramidal neurons were selected on the basis of their characteristic morphology.

For whole-cell recording experiments, glutamate (1 mM) and/or drug perfusion was carried out with a 16-barrel pipette array made from small-diameter (600 $\mu m^2$ i.d.) glass capillary tubing. The pipette array was positioned 100–200 μm from the cell and was moved using a DC actuator. Electronic valves controlled the gravity-induced flow of each solution from a 10 ml syringe to an individual barrel. The solutions from the drug array were changed (100 ms) by altering the array position with a DC actuator (Newport, Irvine, CA).

For experiments using the outside-out patch recording configuration, glass recording electrodes were prepared as described above, and the internal electrode solution contained 115 mM $CsMeSO_3$, 20 mM CsCl, 10 mM HEPES, 2.5 mM $MgCl_2$, 4 mM $Na_2$-ATP, 0.4 mM Na-GTP, 10 mM phosphocreatine, 0.6 mM EGTA, 0.1 mM spermine, 1 mM QX314 (pH 7.2 ± 0.03), 300 ± 2 mOsm. Cells initially were placed into a Petri dish containing the following solution: 119 mM NaCl, 2.5 mM KCl, 2 mM $CaCl_2$, 1.3 mM $MgSO_4$, 2.7 mM $MgCl_2$, 1 mM $NaH_2PO_4$, 26 mM $NaHCO_3$, 11 mM glucose (pH 7.3, 300 ± 2 mOsm). In order to accurately measure deactivation and desensitization kinetics, ultrafast ligand perfusion was carried out using a theta tube constructed from double-barrel borosilicate glass tubing (catalog #TGC200-4;

Warner Instruments, Hamden, CT). The theta tubing was pulled so that the final diameter of each barrel was ~125 μm, and the septum between barrels was ~80 μm. The theta tube position was shifted using a piezoelectric actuator (Burleigh Instruments, Fishers, NY) having a charging time of 0.3 ms. Onset-to-offset time of junction potential measurements (0.1 and 1.0 M NaCl) was 700 μs. Solutions were delivered to the theta tubing using the gravity-induced flow method described above. A high concentration of glutamate (100 mM) was applied to outside-out patches in order to evoke AMPA receptor-mediated currents. The osmolarity of the extracellular solution was adjusted accordingly by reducing NaCl. For recordings from primary culture neurons and acutely isolated hippocampal CA2 and CA3 neurons, the extracellular solution also contained picrotoxin (100 μM) and/or bicuculline (20 μM) to block γ-aminobutyric $acid_A$ receptors and 3-(2-carboxypiperazin-4-yl)-propyl-1-phosphonic acid (CPP; 10 μM) or 2-amino-5-phosphonovaleric acid (APV; 50 μM) to block *N*-methyl-D-aspartate receptors, LY382884 (10 μM) to block kainate receptors, and tetrodotoxin (500 nM) to block synaptic transmission.

### Trafficking Assay

HA-tagged GluR1iQ (Chen et al., 2000; Vandenberghe et al., 2005a) and non-tagged GluR2iR cDNAs driven by a CMV promoter were used to analyze surface expression of GluRs. CHO cells on poly-D-lysine-coated coverslips in 35 mm dishes were transfected with cDNAs encoding GluR (160 ng), γ-subunits (40 ng), and mock vector pcDNA3 (750 ng) using Fugene 6 (Roche). Cells were incubated for 36–48 hr after transfection in 20 mM NBQX. Anti-N-terminal GluR2 (Millipore, MAB397, 1:60) or anti-HA (Covance, 16B12, 1:1000) were diluted in DMEM + 5% FBS were added to the transfected cells at 37°C for 30 min. Cells were briefly washed with HBSS and then fixed with 4% PFA in PBS for 30 min at 4°C, followed by washing in PBS for 15 min twice. Surface-labeled primary antibodies were visualized by anti-mouse IgG conjugated with Alexa 488 (Invitrogen). The cells were then permeabilized with PBS containing 0.1% Triton X-100 and 5% normal goat serum for 30 min at 22°C followed by incubation with anti-C-terminal GluR1 (Millipore, AB1504, 1:200) or anti-C-terminal GluR2/3 (Millipore, AB1506, 1:200). The GluR antibodies labeling internal GluRs were visualized by anti-rabbit IgG conjugated with Alexa 568 (Invitrogen). The stained cells were mounted with Fluoromount-G on slide glasses.

From each coverslip, 12 images were acquired with a DM6000B (Leica) microscope equipped with a ×20 objective and chilled CCD, Spot RTKE (Diagnostic Instruments) at fixed camera gain and exposure time. Optical fields were determined to include GluR-positive cells by observing total GluR labeling (red channel). To eliminate bias, fields were selected by an investigator blinded with respect to surface GluRs labeling. The images were thresholded at a fixed level using Image J 1.37v software (NIH), and cells above threshold were counted.

$BS^3$ surface crosslinking was performed as described (Hall and Soderling, 1997). Briefly, the transfectants (CHO cells) were grown in 35 mm dishes. The cells were washed with PBS, including $Ca^{2+}$ and $Mg^{2+}$ twice, and then incubated with 1 ml of 1 mg/ml of $BS^3$ (Pierce) in PBS at 22°C for 20 m. The cells were washed by PBS containing 100 mM ethanolamine (pH 7.5) twice to quench the excess $BS^3$, followed by harvesting with 100 μl of Laemmli buffer containing 500 mM ethanolamine (pH 7.5). Twenty microliters of the samples was separated by SDS-PAGE and then blotted with anti-GluR1 or anti-GluR2 antibodies.

## SUPPLEMENTAL DATA

The Supplemental Data include six figures and can be found with this article online at http://www.neuron.org/cgi/content/full/59/6/986/DC1/.

## ACKNOWLEDGMENTS

All authors are full-time employees of Eli Lilly and Company. Eli Lilly and Company supported this research.

Accepted: July 25, 2008
Published: September 24, 2008

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